NADH and FADH2, the electron donors for the respiratory chain. NO competes with O2 to slow down electron transfer at the respiratory chain. NO is produced by NO synthases (NOS), with nNOS and eNOS dominating in cardiomyocytes, while the existence of a mitochondrial NOS (mtNOS) is controversial. Since nNOS, eNOS and putative mtNOS are regulated by Ca^{2+}, we speculated that endogenous NO controls respiration during beta-adrenergic stimulation.
Methods and Results: Experiments were performed on murine and guinea-pig mitochondria or cardiomyocytes. In mitochondria, ADP accelerated O_2 consumption and oxidized NADH, while the NO-donor spermine-NONOate inhibited ADP-induced respiration and reduced NADH. Cardiomyocytes were loaded with NO-sensitive DAF-DA, which locates to cytosol and mitochondria, and paced at 0.5 Hz. Isoproterenol plus elevation of stimulation frequency to 5 Hz increased cellular DAF-DA fluorescence by ~12% within 3 minutes, which was abrogated by inhibition or genetic ablation of nNOS, but not eNOS. Dialyzing myocytes with DAF-free pipette solution eliminated >50% of DAF fluorescence, with remaining DAF signals deriving from mitochondria. After beta-adrenergic stimulation, a smaller increase in DAF fluorescence remained, which was abrogated by nNOS KO, but not by Ru360 (1μM in pipette), a blocker of the mitochondrial Ca^{2+} uniporter. The redox states of NADH and FADH2 were dynamically regulated after isoproterenol/5Hz, but not different after nNOS inhibition or KO.
Conclusions: During beta-adrenergic stimulation, most endogenous NO derives from nNOS, but not eNOS, while mtNOS plays no role under these conditions. Although NO inhibits mitochondrial respiration in isolated mitochondria, the endogenous concentrations produced in myocytes during beta-adrenergic stimulation do not affect respiration.

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Two-Photon Fluorescence Lifetime Imaging of Natural Coenzymes in Living Cells as a Function of Oxidative Stress

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Mitochondria play vital roles in energy metabolism, apoptosis, oxidative stress, aging, and neurodegenerative disease [1]. In this contribution, we probe different aspects of cellular response to chemical-induced oxidative stress in living C3H10T1/2 cells using hydrogen peroxide, rotenone, and excess glucose. Using two-photon fluorescence lifetime imaging microscopy (2P-FLIM), we exploit the autofluorescence dynamics of natural coenzymes such as nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD) and flavoproteins as intrinsic biomarkers for oxidative stress. The effects of polarization selectivity in 2P-FLIM measurements are being investigated towards the development of a quantitative, genuine non-invasive 2P-FLIM of patho-physiological changes in living cells. The efficiency of 2P-FLIM cellular autofluorescence for monitoring changes in the metabolic and redox states of the cells is compared with conventional assays such as MitoSOX Red, JC-1, and Rhodamine-123 that are routinely used for oxidative stress studies. Our results help in the collective effort to establish cellular autofluorescence as a natural biomarker for biological and biomedical studies.

1. Heikal, A.A. Intracellular coenzymes as natural biomarkers for metabolic activities and mitochondrial anomalies. *Biomarkers in Medicine*, 4(2): 241-63 (2010).

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Two-Photon Fluorescence Lifetime Imaging for Metabolic Profiling of Cochlear Dysfunction

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More than 120,000 individuals treated with lifesaving antibiotics develop hearing or balance disorders annually. Although research has shown that of the two cochlear cell types, sensory and supporting cells, sensory cells are readily damaged due to age-related hearing loss, acoustic trauma and ototoxins, the reason for this remains unknown. Furthermore, cochlear sensory hair cells can be divided into two types, inner and outer hair cells (IHCs, OHCs). OHCs in the high-frequency region of the cochlea exhibit the greatest sensitivity to the above conditions. To determine if variations in sensory and supporting mitochondrial metabolism account for these differences, two-photon fluorescence lifetime microscopy (FLIM) was used to measure changes in the metabolic reporter molecule NADH in sensory and supporting cells from explanted murine cochleae. Mitochondrial uncouplers, inhibitors and an ototoxic antibiotic, gentamicin (GM), were used to assess high- and low-frequency IHC, OHC and supporting cell mitochondrial metabolism. Chemically induced changes in metabolic state resulted in a reorganization of specific NADH lifetimes into altered subcellular fluorescence lifetime pools. Variations in NADH intensity and average NADH lifetime were greatest in high-frequency OHCs. Pretreatment with GM significantly increased NADH intensity in high-frequency sensory cells but not supporting cells. Treatment with GM significantly increased the average NADH fluorescence lifetime within IHCs but not OHCs. GM also caused a significant increase of NADH concentration in OHCs, not IHCs. These results demonstrate: differences between sensory and supporting cell metabolism; GM alters mitochondrial metabolism; and IHCs and OHCs display differing metabolic effects when exposed to GM. Such fundamental differences between sensory and supporting cell mitochondrial metabolism indicate differing metabolic changes during antibiotic exposure. Understanding these antibiotic-induced metabolic changes may explain the ototoxic effects of these drugs which is crucial for preventing and treating numerous auditory deficits and diseases.

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Endogenous Differences in Cochlear Sensory and Supporting Cell Mitochondrial Metabolism Bias Free Radical Production during Ototoxin Exposure

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Aminoglycosides, including gentamicin (GM), are the most frequently used antibiotics in the world despite irreversible cochlear damage and hearing loss associated with their use. Although there are numerous causes of deafness, reactive oxygen species (ROS) are key regulators of multiple pathologies including: ototoxicity, noise-induced and age-related hearing loss. Unfortunately, the source of these cell-damaging ROS remains controversial. Given that ROS are normal byproducts of ATP synthesis, intrinsic differences in cochlear sensory (inner and outer hair cell, I/OHC) and supporting (pillar) cell mitochondrial metabolism may explain why high-frequency OHCs are profoundly sensitive to a host of challenges. Mitochondrial metabolism was compared in low-frequency and high-frequency IHCs, OHCs and pillar cells from acutely cultured cochlear explants. Intensity-based changes in the metabolic intermediate, nicotinamide adenine dinucleotide (NADH), were used to measure endogenous, mitochondrial toxin and GM-induced differences in sensory and supporting cell mitochondrial metabolism. Sensory cell mitochondrial metabolism was significantly enhanced relative to supporting cells. Despite similar amounts of mitochondria in IHCs and OHCs, endogenous levels of NADH were greatest in high-frequency OHCs. Metabolic profiling of NADH metabolism revealed basal turn, high-frequency OHCs to be metabolically responsive to a number of changes in their microenvironment including metabolic toxins and GM, while high-frequency IHCs, low-frequency I/OHCs and, pillar cells are substantially less sensitive. DHR-123 was used to detect sensory and supporting cell ROS production during GM exposure. Within 30 minutes of GM application, ROS are dramatically and specifically increased in high-frequency sensory cells. GM-induced changes in mitochondrial metabolism and cell-damaging free radical production were greatest in high-frequency OHCs. This metabolic predisposition biases basal turn OHC responses to a variety of cochlear insults, particularly those involving energy metabolism and ROS production.

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Electron Transport Activity in Embryonic Hearts Requires the Formation of Supercomplexes

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The heart is the first functional organ in a vertebrate embryo. Mitochondria appear to be important to heart development and closing the permeability transition pore (PTP) between embryonic day (E) 9.5 and E11.5 drives the maturation of mitochondrial structure and regulates myocyte differentiation. These data also suggested that closure of the PTP is associated with changes of individual complexes (Cx) of the electron transport chain (ETC), and the objective here was to further define these changes.
We assessed the activity of the Cx- 1, Cx-2 and Cx-3 of the ETC of C57BL/6N (wildtype (WT)) and cyclophilin D knockout (CypD KO) mice during embryonic development from E9.5 to E13.5 with a Clark-type oxygen electrode and enzymatic assays. Our results show that at E9.5 mitochondrial oxygen consumption appears uncoupled and there is no difference between state 2, 3 and 4 respiration. In contrast, oxygen consumption in E9.5 CypD KO embryos